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Effects of visfatin on brown adipose tissue energy regulation using T37i cells

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ABSTRACT

The role of brown adipose tissue (BAT) in pathological states of energy homeostasis and impaired adipocyte function, such as obesity has been a major area of research interest in recent years. Herein, we sought to determine the direct effects of adipokines, visfatin and leptin on BAT thermogenesis.

The effects of mouse recombinant visfatin, nicotinamide mononucleotide (NMN) and leptin with or without FK866 were studied on differentiated T37i cells. Treated cells were analyzed for key genes and proteins regulating BAT [UCP-1, PRD1-BF1-RIZ1 homologous domain-containing 16 (PRDM-16), PPARgamma-coactivator-1alpha (PGC-1α) and receptor-interacting protein 140 (RIP-140)] using quantitative PCR and western blot analysis. Data is presented as mean *P*-values.

Both visfatin and leptin had significant concentration dependent effects on thermogenesis in brown preadipocytes and at physiological levels, increased uncoupling protein-1 (UCP-1) levels in brown adipocytes. These effects of visfatin were similar to that of nicotinamide mononucleotide (NMN), further strengthening the enzymatic role of visfatin. We also showed that leptin induced UCP-1 mRNA expression and protein production appears to be mediated by visfatin. High concentrations of both visfatin and leptin led to a dramatic decrease in UCP-1 protein levels, supporting the notion that visfatin levels are raised in obesity and that obese people have reduced BAT activity, plausibly through a reduction in UCP-1 levels. Additionally, we found differential regulation of key brown adipogenic genes, specifically, PRD1-BF1-RIZ1 homologous domain-containing 16 (PRDM-16), PPARgamma-coactivator-1alpha (PGC-1α) and receptor-interacting protein 140 (RIP-140) by visfatin. Our observations provide novel insights in the potential actions of visfatin in BAT.

1. Introduction

Adipose tissue (AT) functions as a key energy metabolic regulator [1]. White adipose tissue (WAT) serves as an energy reservoir, whilst brown adipose tissue (BAT) is involved in β-adrenergically mediated thermogenesis [2]. Recent studies have implicated the importance of this metabolic activity in BAT in states of insulin resistance and obesity (inversely correlated with body mass index and age) [3]. BAT has been regarded as a key target in developing anti-obesity treatment. Although stimulants of the adrenergic system are excellent agents for enhancing BAT activity, their use has been limited by harmful cardiovascular side-effects. A safer approach employing endogenous molecules, specifically,

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AT derived molecules (adipokines), as potential thermogenic agents stimulating BAT is currently under focus, being investigated extensively [4].

Studies have elucidated the link between adipokines and AT function. Dysmetabolic states are characterized by altered circulating adipokine levels e.g. leptin and visfatin, levels of which are raised in obesity and metabolic syndrome, [5,6] influencing BAT function. Enriori et al. had demonstrated that central and peripheral administration of leptin significantly increased BAT energy expenditure *via* increased expression of mitochondrial uncoupling protein-1 (UCP-1) [7]. Other researchers have also demonstrated that in brown adipocytes undergoing differentiation, visfatin (intracellular and extracellular) levels are increased [8]. However, no studies have been to date conducted to elucidate the direct effects of visfatin (pre-B cell colony-enhancing factor or Nicotinamide phosphoribosyltransferase) a crucial energy regulator, on brown adipocytes. Moreover, we have previously demonstrated both *in vivo* and *ex vivo* regulation of visfatin production by leptin in WAT [9]. With this in mind, we sought to investigate the effects of leptin and visfatin in differentiated T37i cells, mitochondrial respiration and key genes involved in brown adipocyte energy regulation.

2. Materials and methods

We studied the effect of mouse recombinant visfatin (Axxora, Nottingham, UK), nicotinamide mononucleotide (NMN) (Sigma-Aldrich, Gillingham, UK) and leptin (PeproTech, Rocky Hill, NJ, USA) with or without FK866 [a highly specific noncompetitive inhibitor of nicotinamide phosphoribosyltransferase (AxonChem, Groningen, Netherlands)] on differentiated T37i cells (a kind gift provided by Dr Marc Lombes, INSERM, Paris, France) cultured in standard DMEM:HAM's F12 medium (Invitrogen, Paisley, UK) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 20 mM HEPES and grown at 37 °C in a humidified atmosphere with 5% CO_2. Cells were differentiated with 2 nM triiodothyronine (Sigma-Aldrich, Gillingham, UK) and 20 nM insulin (Invitrogen, Paisley, UK) for 8 days [10,11]. The treated cells were analysed for key genes and proteins regulating BAT conversion [UCP-1, PRD1-BF1-RIZ1 homologous domain-containing 16 (PRDM-16), PPARgamma-coactivator-1alpha (PGC-1α) and receptor-interacting protein 140 (RIP-140)] using quantitative PCR and Western blot analysis [12,13]. Isoproterenol (Sigma-Aldrich, Gillingham, UK) was used as a positive control in some experiments. Lipid accumulation was performed in these treated cells by using Oil Red O (Sigma-Aldrich, Gillingham, UK) staining.

2.1. Bioenergetic analysis of brown adipocytes

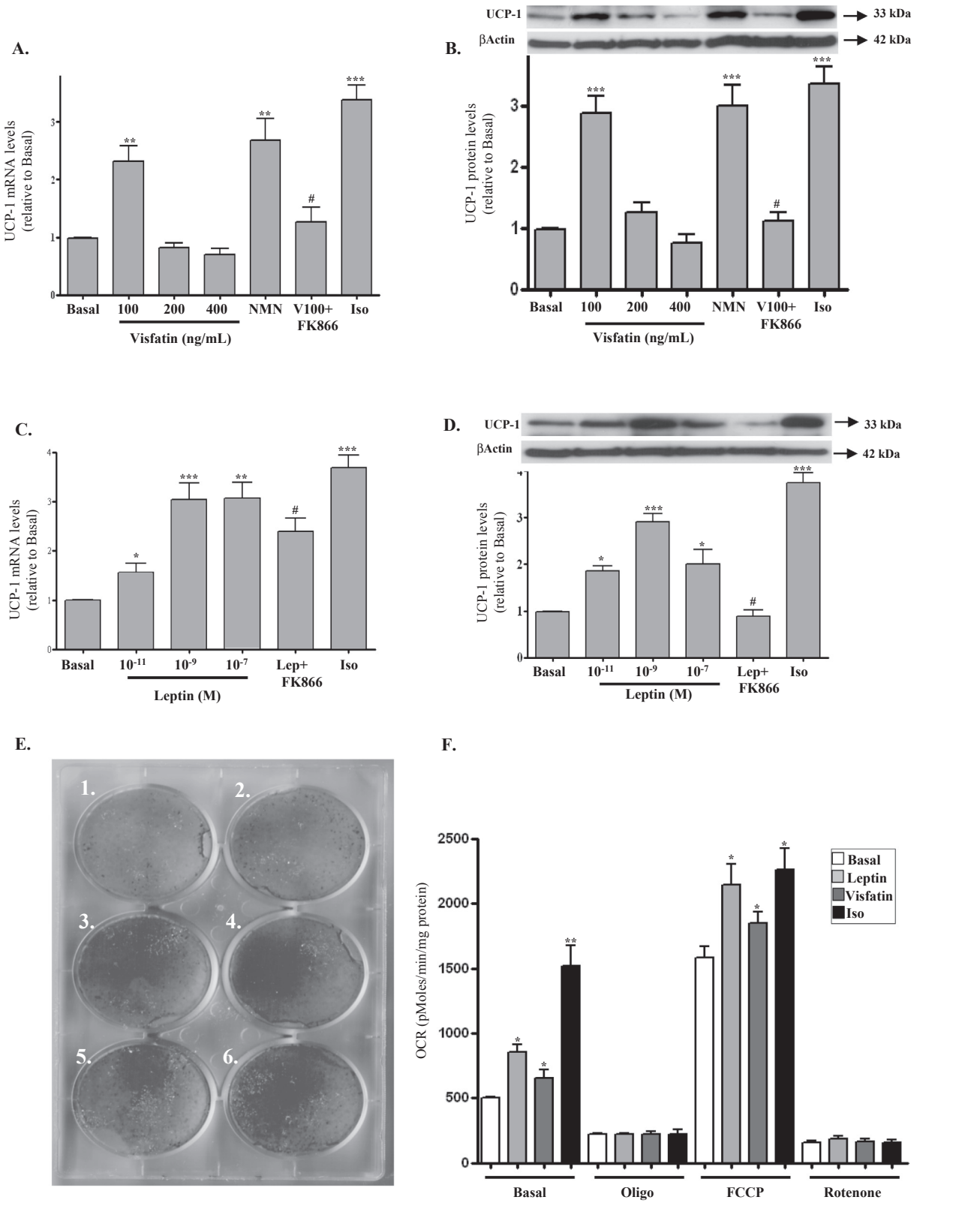
The XF24 Extracellular/Flux Analyser (Seahorse Biosciences, North Billerica, MA, USA) was employed for bioenergetic analysis of T37i differentiated cells. All the chemicals required for these experiments were supplied by Seahorse Biosciences, North Billerica, MA, USA. The XF24 Extracellular/Flux Analyser measures oxygen consumption rate (OCR) in a 24-well format by sensing changes in oxygen content (in a 7 μl volume) above the plated cells with a fluorescence biosensor. T37i cells were seeded at a density of 3×10^4 cells/well/500 μl of Agilent Seahorse XF Assay Medium (Agilent Technologies) in an extracellular flux 24-well cell culture plate and incubated at 37 °C/5% Co_2 for 8 days. At the end of day 7, cells were stimulated with either visfatin (100 ng/mL) or leptin (10^{-9} M) or isoproterenol and cultured for a further 24 h. Assays were initiated by removing the growth medium and replacing it with pre-warmed assay buffer medium. The microplates were incubated at 37 °C for 30 min to equilibrate temperature and pH prior to OCR measurements. The XF24 microplate was then transferred to a temperature-controlled (37 °C) Seahorse analyzer where it was subjected to a further 10-minute equilibration period and 4 assay cycles, each comprising a 1-minute mix, 2-minute wait and 3-minute measure period cycle. Following this, Oligomycin A (1 μM) was added by automatic pneumatic injection to inhibit ATP synthase activity and thus approximate the proportion of respiration used to drive ATP synthesis (coupling efficiency). After 4 assay cycles, 7.5 μM carbonyl cyanide 4 trifluoromethoxy-phenylhydrazone (FCCP) was added. Each experimental trace was ended following addition of oligomycin A (5 μM) and rotenone (5 μM). At the end of the incubation period, the plates were used to assess protein concentration in each well by BCA protein assay. OCR (pmol/min) was normalised with protein content.

2.2. T37i cell culture and differentiation

T37i cells (a kind gift provided by Dr Marc Lombes, INSERM, Paris, France) were cultured in standard DMEM:HAM's F12 medium (Invitrogen, Paisley, UK) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 20 mM HEPES and grown at 37 °C in a humidified atmosphere with 5% CO_2. Differentiation into mature brown adipocytes was achieved under standard conditions by incubating sub-confluent undifferentiated T37i cells with 2 nM triiodothyronine [T3] (Sigma-Aldrich, Gillingham, UK)] and 20 nM insulin (Invitrogen, Paisley, UK) for 8 days. At day 8 of differentiation, the cells were harvested for RNA (RT-PCR) and protein (Western blot). T37i cells were cultured in the presence of only differentiation stimuli (insulin + T3) for the control group and differentiation stimuli with or without visfatin (Axxora, Nottingham, UK), nicotinamide mononucleotide (NMN) (Sigma-Aldrich, Gillingham, UK) or FK866 (AxonChem, Groningen, Netherlands) for the treatment group with two incubation time points as mentioned in the figure legend (Fig. 1). Before stimulation, the differentiated cells were cultured overnight in the same media in the absence of serum. For UCP-1 mRNA expression and protein production studies, cells were incubated with media supplemented with 1 μM isoproterenol for 6 h prior to stimulation with peptides. The treated cells were analysed for key genes and proteins regulating brown adipose tissue conversion [UCP-1, PRD1-BF1-RIZ1 homologous domain-containing 16 (PRDM-16), PPARgamma-coactivator-1alpha (PGC-1α) and receptor-interacting protein 140 (RIP-140)] using quantitative RT-PCR and Western blot analysis.

2.3. RNA isolation and Real-Time quantitative reverse transcription polymerase chain reaction

Total RNA was extracted using the QIAGEN RNeasy Mini Kit (Qiagen Ltd, West Sussex, UK), DNAse treated and reverse-transcribed into cDNA using a reverse transcription kit according to the manufacturer's instructions (Fermentas Life Sciences, York, UK). The sequences for the sense and antisense primers (respectively) are listed in Table 1. SYBR Green® real-time PCR was performed on a Biorad IQ5 realtime PCR (Bio-Rad, Hertfordshire, UK) using the primers listed above. PCR was performed using 2.5 μl cDNA in 5.5 μl PCR SYBR Green-1 Light Cycler Master Mix (Biogene, Cambridgeshire, UK), and 1 μl each of sense and antisense primers (primer sequences as mentioned in Table 1 below). A series of three dilutions for each cDNA was used to ensure linear amplification and to measure primer efficiency. Protocol conditions consisted of denaturation of 95 °C for 60 secs, followed by 40 cycles of 94 °C for 1 sec, 60 °C for 8 sec, and 72 °C for 15 sec, followed by melting-curve analysis. For analysis, expression of genes of interest were normalised against the expression of the housekeeping gene GAPDH. Negative controls for all the reactions included preparations lacking cDNA or RNA-lacking reverse transcriptase in place of the cDNA. The relative mRNA levels were expressed as a ratio using the "2-Δct method" for comparing relative expression results between treatments in real-time PCR[1]. The PCR products from all samples were purified from the 1% agarose gel using the QIAquick Gel Extraction Kit (Qiagen Ltd, West Sussex, UK). PCR products were then sequenced in an automated DNA sequencer, and the sequence data were analyzed using



(caption on next page)

Blast Nucleic Acid Database Searches from the National Centre for Biotechnology Information, confirming the identity of our products.

Semi-quantitative PCR analysis was employed for the identification of long form of leptin receptor (Ob-Rb) in T37i cells. Following RNA extraction and cDNA conversion, using specific primers for Ob-Rb gene (mentioned in the Table 1 below) PCR was carried out for 40 cycles comprising of denaturation at 95 °C for 1 min, annealing at 60 °C for 30 sec, and extension at 72 °C for 1 min. Amplification was terminated following a final extension step at 72 °C for 10 min. PCR products were separated by agarose electrophoresis (1.5% agarose gel), stained with

Fig. 1. Serum-starved differentiated T37i cells were pre-incubated with or without FK866 (10 μM). On treatment with or without visfatin (100–400 ng/mL) or NMN (100 μM) or isoproterenol (1 μM) for 4 h, mRNA levels of (A) UCP-1 was analyzed by real-time PCR and normalized with the housekeeping gene GAPDH. Similarly, serum-starved differentiated T37i cells were treated for 24 h, protein expression levels of (B) UCP-1 was analyzed by western blot analyses and densitometric analyses of UCP-1, normalized to β-actin and expressed as a fold increase over basal. Experiments were performed in triplicates. The values represented are relative to basal. $^{**}P < 0.01$, $^{*}P < 0.05$ vs. basal, $^{\#}P < 0.01$ vs. visfatin (100 ng/mL) only treated, n = 6 per group. Likewise, serum-starved differentiated T37i cells were pre-incubated with or without FK866 (10 μM). On treatment with or without leptin (10^{-11}, 10^{-9} and 10^{-7} M) or isoproterenol (1 μM) for 4 h, mRNA levels of (C) UCP-1 was analyzed by real-time PCR and normalized with the housekeeping gene GAPDH. Similarly, serum-starved differentiated T37i cells were treated for 24 h, protein expression levels of (D) UCP-1 was analyzed by western blot analyses and densitometric analyses of UCP-1, normalized to β-actin and expressed as a fold increase over basal. Experiments were performed in triplicates. The values represented are relative to basal. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ vs. basal, $^{\#}P < 0.01$ vs. leptin (10^{-9} M) only treated n = 6 per group. T37i cells were differentiated with insulin and T3 (6 days) for control group and differentiation stimuli with or without visfatin (100–400 ng/mL) or NMN (100 μM) or leptin (10^{-11}, 10^{-9} and 10^{-7} M) for treatment group for 2 days. One set of treatment group was pre-incubated with FK866 (10 μM). (E) Representative scanned images of Oil Red O stained brown adipocytes [1] and [2] Control (undifferentiated) [3] Control (differentiated) [4] Visfatin(100 ng/mL) [5] Vis(100 ng/mL) + FK866(10 μM) [6] NMN(100 μM).(F) Mitochondrial respiration [oxygen consumption rate (OCR)] in differentiated brown adipocytes (day 6) was assessed using the XF24 Extracellular/Flux Analyser. A representative graph of the OCRs of untreated, visfatin, leptin and isoproterenol treated cells in their basal states and on treatment with oligomycin, FCCP and rotenone used to demonstrate the specific components of the respiratory chain. Experiments were performed in triplicates. The values represented are relative to basal. $^{**}P < 0.01$, $^{*}P < 0.05$ vs. untreated cells (control of that particular group), n = 6 per group.

Table 1
List of genes and sequences for the sense and antisense primers.

Gene/product size (bp)	Sense primer	Antisense primer
UCP-1 (83)	5′-ggcctctacgactcagtcca-3′	5′- taagccggctgagatcttgt-3′
PRDM-16 (180)	5′- atgcgaggtctgccacaagt-3′	5′- ctgccaggcgtgtaatggtt-3′
RIP-140 (173)	5′-tgcggatacttccacaggtc-3′	5′-gcattcctcacagccaacag-3′
PGC-1 alpha (163)	5′-tgcagccaagactctgtatg-3′	5′-attggtcgctacaccacttc-3′
GAPDH (185)	5′-gagtcaacggatttggtcgt-3′	5′-gacaagcttcccgttctcag-3′
Ob-Rb (182)	5′-aggcgcagcctgtattgtcc-3′	5′-cacgttggtggcgagtcaag-3′
CtBP1 (171)	5′-ttgggcatcattggactaggt-3′	5′-taacgcagtcactgtggaaga-3′
CtBP2 (162)	5′-atagaacgatctctgggcctg-3′	5′-aatgcaccttgctctatctgc-3′
BMP-7 (99)	5′-cttggctggcaggactggat-3′	5′-gtctggacgatggcgtggtt-3′

ethidium bromide, and visualized under UV light.

2.4. Western blot analysis

For UCP-1, PRDM-16 and BMP-7 protein analyses, following differentiation, T37i cells were incubated with media supplemented with 1 μM isoproterenol for 6 h prior to stimulation with only differentiation stimuli (insulin + T3) for the control group and differentiation stimuli [with or without visfatin, leptin, NMN or FK866] for the treatment group with various incubation time points as mentioned previously. Cells were then lysed with Laemmli buffer [5 M urea, 0.17 M SDS, 0.4 M dithiothreitol and 50 mM Tris-HCl (pH 8.0)], mixed, sonicated, boiled, centrifuged (5000 rpm for 2 min), and stored at −80 °C until use. Twenty micrograms of each sample were separated on a 10% Sodium Dodecyl Sulfate (SDS)-polyacrylamide gel, and electro-blotted onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA). The PVDF membrane was then incubated with 5% Bovine Serum Albumin (BSA) (Sigma-Aldrich, Gillingham, UK) in 1 M Trizma/base, 1.54 M NaCl, 0.05% Tween 20 (Tris buffered solution plus Tween 20, TBST, pH 7.4) for one hour at room temperature, and then exposed overnight at 4 °C to TBST containing UCP-1 primary antibody [Abcam, Cambridge, UK (AB23841, dilution 1:1000)]. The membranes were then washed thoroughly for 60 min with TBS/0.1% Tween before incubation with anti-mouse secondary antibody, horseradish-peroxidase-conjugated Ig (1:2000) (Dako Ltd, Cambridge, UK) for 1 h at room temperature. Antibody complexes were visualized using chemiluminescence (ECL; GE Healthcare, Little Chalfont, UK). The densities were measured using a scanning densitometer coupled to scanning software ScionImage™ (Scion Corporation, Frederick, Maryland, USA). Standard curves were generated to ensure linearity of signal intensity over the range of protein amounts loaded into gel lanes. Comparisons of densitometric signal intensities for proteins of interest were made only within this linear range.

2.5. Oil Red O stain

As mentioned previously, T37i cells were differentiated with insulin and T3 for the control group and differentiation stimuli with or without visfatin, leptin, NMN or FK866 for the treatment group for 8 days. Following differentiation, the cells were washed with PBS and fixed with 10% formalin (Sigma-Aldrich, Gillingham, UK). This was followed by washes with distilled water and 60% isopropanol (Sigma-Aldrich, Gillingham, UK). The cells were allowed to air dry, Oil Red O (Sigma-Aldrich, Gillingham, UK) working solution was added and incubated at room temperature for 10 min. The cells were washed 4 times with distilled water and images were acquired.

2.6. Statistics

Data were analyzed by Mann-Whitney *U* test or Friedman's ANOVA (*post hoc* analysis, Dunn's test) according to the number of groups compared. All statistical analyses were performed using SPSS version 18.0 (SPSS, Inc., Chicago, IL). $P < 0.05$ was considered significant.

3. Results

Visfatin and leptin significantly increased UCP-1 mRNA and protein expression in differentiated T37i cells (Fig. 1A–D). Visfatin (100 ng/mL) showed the maximal response in contrary to visfatin (400 ng/mL), which decreased UCP-1 levels. NMN (100 μM) showed a comparable increase of UCP-1 levels to visfatin (100 ng/mL). Pre-incubation of FK866 (10 μM) abolished visfatin (100 ng/mL) induction of UCP-1 (Fig. 1A, B). Leptin increased UCP-1 protein levels (maximal response at 10^{-9} M), which was significantly negated by pre-incubating with FK866 (10 μM) (Fig. 1C, D). Following differentiation protocol, T37i cells were serum starved for 12 h and treated with or without visfatin (100, 200 and 400 ng/mL,), NMN (100 μM) and FK866 (10 μM) for two time points (4 and 24 h). Visfatin (100 ng/mL) induced maximal expression of PRDM-16 mRNA at 4 h. This effect of visfatin was not negated when pre-incubated with FK866 (10 μM). NMN (100 μM) induced a significant increase with comparable potency to visfatin (100 ng/mL) (Fig. 2A). Furthermore, PGC-1α mRNA expression was significantly increased in a concentration dependent manner by visfatin (maximal response at 400 ng/mL). This effect was negated by pre-incubating FK866 (10 μM). Once again, NMN (100 μM) induced a significant increase with comparable potency to visfatin (400 ng/mL) (Fig. 2B). Also, visfatin (100 ng/mL) significantly decreased RIP-140 mRNA expressions (Fig. 2C). Interestingly, visfatin (400 ng/mL), at 4 h, resulted in a significant increase in RIP-140 mRNA expression. However, no effect was observed with NMN (100 μM) treatment. Pre-incubation with FK866 (10 μM) resulted in a non-significant decrease of visfatin (400 ng/mL) induced RIP-140 mRNA expression (Fig. 2C). Leptin

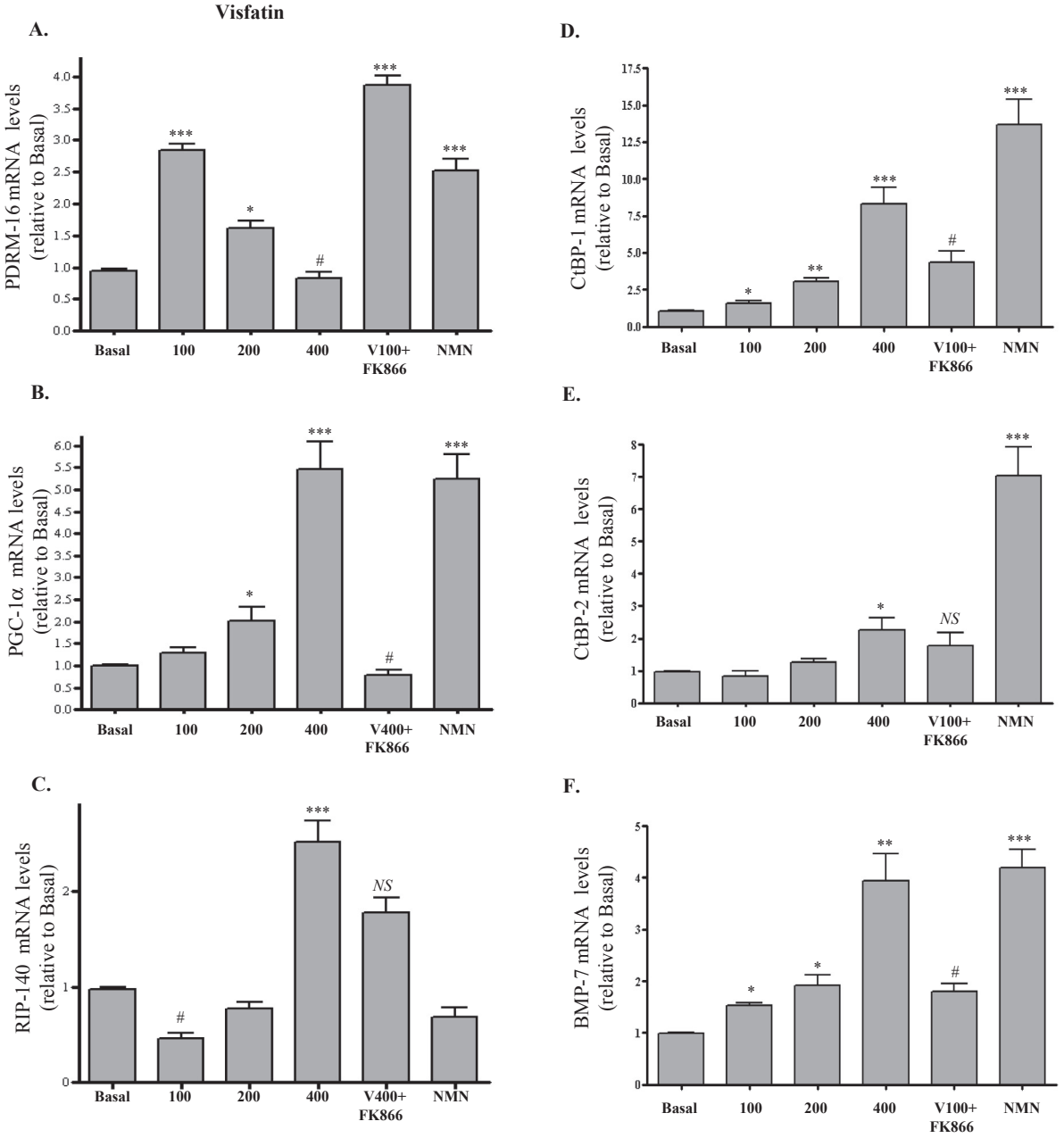


Fig. 2. Serum-starved differentiated T37i cells were pre-incubated with or without FK866 (10 μM). On treatment with or without visfatin (100–400 ng/mL) or NMN (100 μM) for 4 h, mRNA levels of (A) PDRM-16, (B) PGC-1α, (C) RIP-140, (E) CtBP-1, (F) CtBP-2 and (G) BMP-7 were analyzed by real-time PCR and normalized with the housekeeping gene GAPDH. Experiments were performed in triplicates. The values represented are relative to basal. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ vs. basal, $^{\#}P < 0.01$ vs. visfatin (100 or 400 ng/mL) only treated, *NS*- Non-Significant vs. basal, n = 6 per group.

increased mRNA expression levels of PDRM-16 and PGC-1α; however, similar to visfatin, the maximal response dose of leptin i.e. 10^{-9} M, decreased RIP-140 mRNA expression levels (data not shown). Following differentiation protocol, T37i cells were serum starved overnight, and treated with or without leptin (10^{-7}, 10^{-9} and 10^{-11} M) for various time points (4–24 h – data not shown). Leptin significantly increased mRNA expression and protein production of visfatin (Maximal response was at 4 h and leptin 10^{-7} M) (Fig. 4A, B). Finally, functional assays showed that visfatin (100 ng/mL) caused a significant increase in lipid accumulation (Oil Red O stain) in pre-adipocytes and oxygen consumption (mitochondrial respiration) in mature brown adipocytes (Fig. 1E, F).

3.1. Visfatin induces visfatin mRNA expression levels of brown adipogenic genes- CtBP-1, CtBP-2 and BMP-7

In order to evaluate overall visfatin induced effects in brown adipogenesis, we sought to determine the effects of visfatin on key genes including CtBP-1 (C-terminal-binding protein 1), CtBP-2 and BMP-7 (Bone morphogenetic protein 7). Visfatin (400 ng/mL) showed the maximal response in up-regulating all three genes, comparable with NMN (100 μM). However, it is interesting to note that pre-incubation of FK866 (10 μM) abolished visfatin (400 ng/mL) induced CtBP-1 and BMP-7 mRNA expression levels (Fig. 2D and F), failed to do so with CtBP-2 mRNA expression (Fig. 2E).

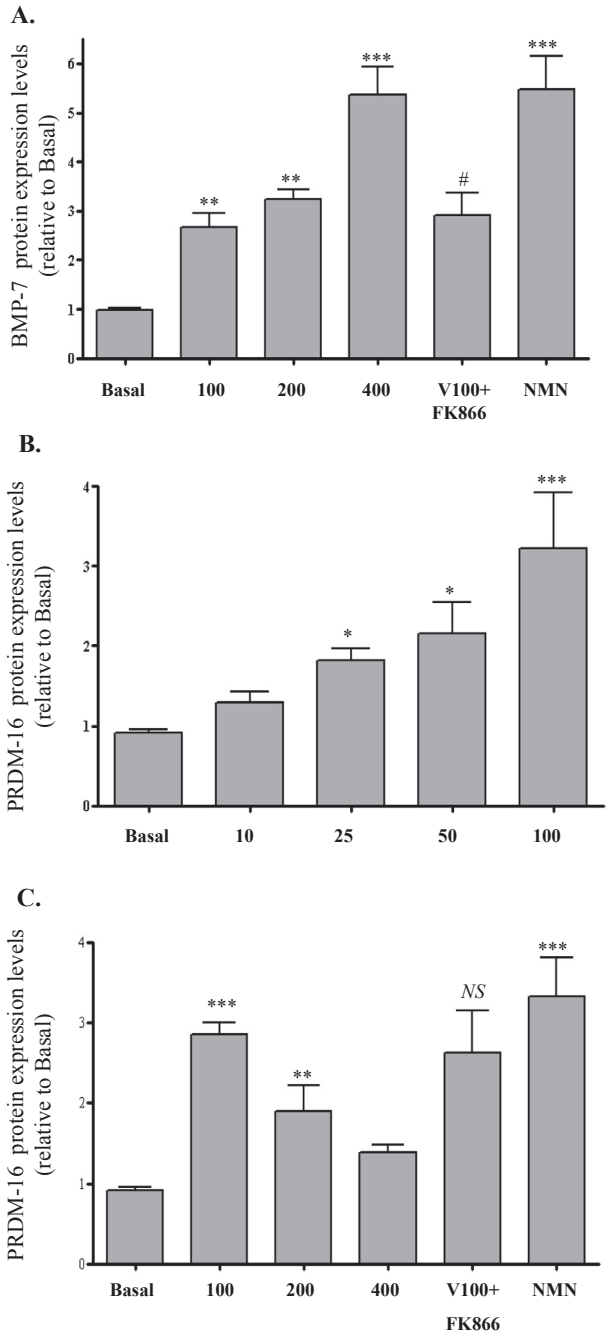


Fig. 3. Serum-starved differentiated T37i cells were pre-incubated with or without FK866 (10 μM). On treatment with or without visfatin (100–400 ng/mL) or NMN (100 μM) for 24 h, protein levels of (A) BMP-7 and (C) PRDM-16 were analyzed by western blot analyses and normalized with the housekeeping protein GAPDH. Similar experiments were performed with concentration dependent visfatin (10, 25, 50 and 100 ng mL) for 24 h and protein levels of (B) PRDM-16 were analyzed by western blot analyses and normalized with the housekeeping protein GAPDH. Experiments were performed in triplicates. The values represented are relative to basal. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ vs. basal, $^{\#}P < 0.01$ vs. visfatin (100 or 400 ng/mL) only treated, *NS*- Non-Significant vs. basal, n = 6 per group.

3.2. Visfatin increases protein expression levels of BMP-7 and PRDM-16 in differentiated T37i cells

Visfatin concentration dependently increased protein expression levels of BMP-7 at 24 h, promptly decreased by pre-incubation with FK-866. This increase induced by visfatin (400 ng/mL) was comparable to the effects of NMN (100 uM) (Fig. 3A). However, in contrary, visfatin (100 ng/mL) showed a significant increase in PRDM-16 protein levels and pre-incubation with FK-866 did not cause any significant effect (Fig. 3C). To further study the concentration dependent effects of visfatin on protein expression levels of PRDM-16, we employed additional doses of 10, 25 and 50 ng/mL of visfatin. The maximal response was observed at 100 ng/mL of visfatin (Fig. 3B).

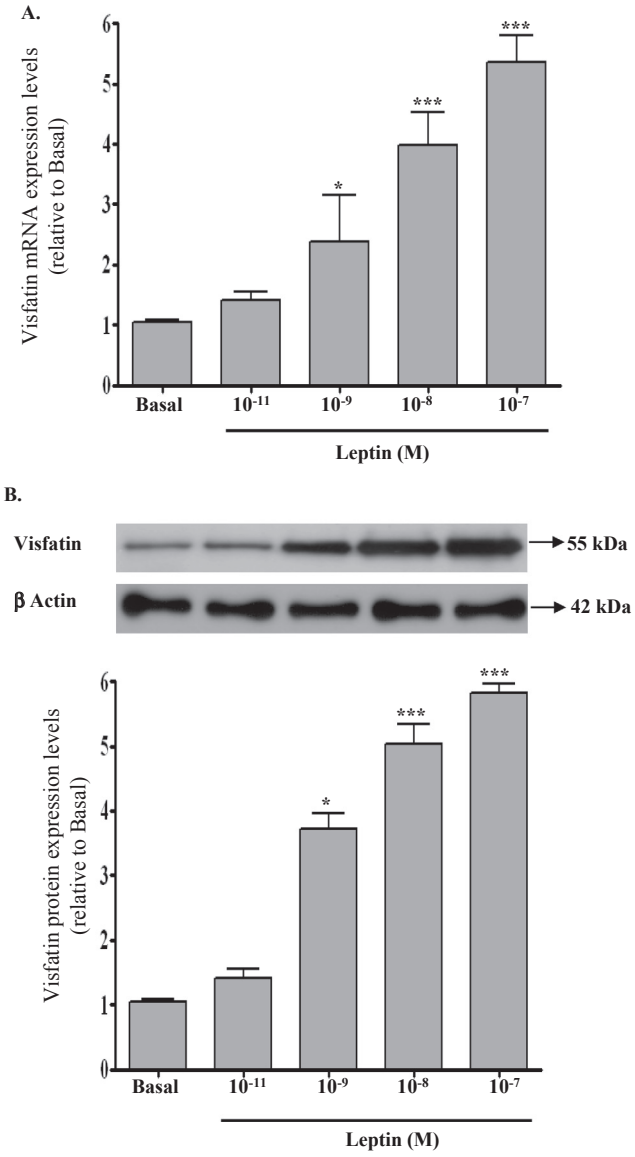


Fig. 4. Following time optimisation studies, serum-starved differentiated T37i cells were treated with or without leptin (10^{-11}, 10^{-9} and 10^{-7} M) for 4 h; (A) mRNA levels of visfatin were analyzed by real-time PCR and normalized with the housekeeping gene GAPDH. Experiments were performed in triplicates. The values represented are relative to basal. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ vs. basal, n = 6 per group. Similar treatments were done with leptin for 24 h, (B) protein expression levels of visfatin were analyzed by western blot and densitometric analyses normalized to β-actin and expressed as a fold increase over basal. Experiments were performed in triplicates. The values represented are relative to basal. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ vs. basal, n = 6 per group.

3.3. Leptin induces visfatin mRNA and protein expression levels in a concentration dependent manner

We used leptin in concentration ranges of 10^{-7}–10^{-11} M in

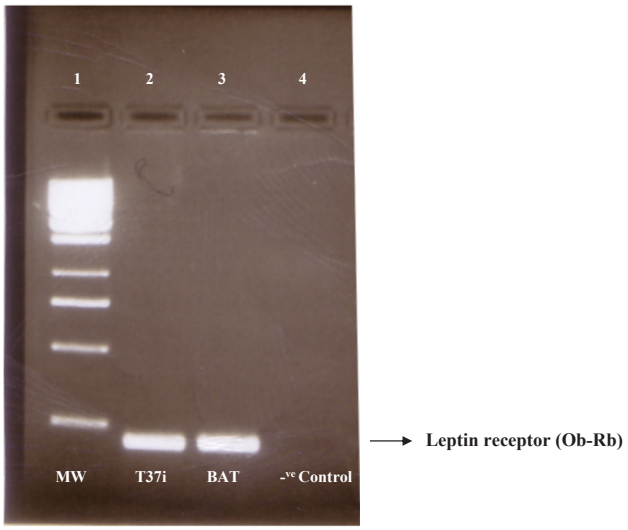


Fig. 5. RT-PCR amplification of Ob-Rb gene. Lane 1, DNA ladder marker; lane 2, cDNA from T37i cells; lane 3, cDNA from mouse BAT; lane 4, $-^{ve}$ control (RT $-^{ve}$ – cDNA preparation lacking reverse transcriptase).

accordance with studies by others [14,15], to include physiological and pathological concentrations [2,3]. Leptin increased visfatin mRNA expression and protein expression levels in T37i cells concentration dependently with a maximum response at leptin 10^{-7} M (Fig. 4A and B).

3.4. Identification of Ob-Rb receptor in T37i cells and mouse BAT

We employed RT-PCR analysis and gene specific primers to validate the presence of Ob-Rb gene in T37i cells and BAT (Fig. 5) in line with previous reports [4].

4. Discussion

We present novel data indicating that visfatin significantly increased oxygen consumption (mitochondrial respiration) and lipid accumulation in differentiated T37i cells. Furthermore, visfatin, at physiological levels, significantly increased UCP-1 and PDRM-16 and decreased RIP-140 levels in T37i cells. Interestingly, FK-866 had no effect on visfatin induced PDRM-16 up-regulation. This may be due to the involvement of an undefined visfatin receptor. Also, this may be explained by the observation that eNampt (visfatin) blocks macrophage apoptosis through activation of IL-6/STAT3 pathway. This effect was not blocked by FK866, suggesting a non-enzymatic mechanism of action [16]. Moreover, like others, [7] we found that leptin significantly increased oxygen consumption and significantly increased UCP-1 levels in T37i cells. Interestingly, higher concentrations of leptin resulted in a significant decrease in UCP-1 levels in T37i cells. Importantly, we showed that leptin induced UCP-1 mRNA expression and protein production appears to be mediated by visfatin. UCP-1 drives BAT thermogenesis, PDRM-16 promotes brown adipogenesis and BAT thermogenesis *via* increased stimulation of UCP-1 [17] whereas RIP-140 is an important co-repressor and inhibitor of brown adipogenesis and BAT thermogenesis [18]. However, higher concentrations of visfatin had opposite effects. This supports the observations that visfatin levels are raised in obesity [5] and that obese people have reduced BAT activity, [19] plausibly through a reduction in UCP-1 levels. Others were able to demonstrate that visfatin was preferentially expressed in mature adipocytes and that this expression was higher in brown adipose tissue of rodents compared to other fat depots following biopsies [20]. The situation was different in obese humans where visfatin expression was found to be equivalent between white and brown or brite adipocytes *in vivo* and *in vitro* supporting that BAT thermogenic properties are blunted during obesity [20]. Interestingly, we also found that visfatin concentration dependently increased PGC-1α (a strong inducer of UCP-1) mRNA expression in T37i cells. PGC-1α promotes BAT thermogenesis but not brown adipogenesis [21]; additionally, we elucidated PGC-1α independent UCP-1 gene induction. Studies have implicated a hyperglycemic state of impaired glucose metabolism induced by PGC-1α inhibiting insulin signaling and glucose utilization [22]. Taken together; our novel findings potentially suggest that visfatin could induce insulin resistance in dysmetabolic states such as obesity and diabetes *via* PGC-1α without a marked change in UCP-1 levels. However, further studies are required in brown adipocytes and *in vivo*.

5. Conclusion

In conclusion, our significant observations provide novel insights with respect to the potential actions of visfatin in brown adipocytes using T37i cell line. Future *in vivo* research should also seek to clarify whether visfatin could moderate thermogenesis and adipogenesis, and whether it would be beneficial in the management of obesity and its dysmetabolic sequelae.

Conflict of interest

The authors declare no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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